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Citation for published version:

Fay, P, Cook, C, Wijesiriwardana, N, Tore, G, Comtet, L, Carpentier, A, Shih, B, Freimanis, G, Haga, IR & Beard, PM 2020, 'Madin-Darby bovine kidney (MDBK) cells are a suitable cell line for the propagation and study of the bovine poxvirus lumpy skin disease virus', *Journal of Virological Methods*, pp. 113943. <https://doi.org/10.1016/j.jviromet.2020.113943>

Digital Object Identifier (DOI):

[10.1016/j.jviromet.2020.113943](https://doi.org/10.1016/j.jviromet.2020.113943)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Publisher's PDF, also known as Version of record

Published In:

Journal of Virological Methods

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Madin-Darby bovine kidney (MDBK) cells are a suitable cell line for the propagation and study of the bovine poxvirus lumpy skin disease virus

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ARTICLE INFO

Keywords:

Lumpy skin disease virus
Poxvirus
Capripoxvirus
Neutralization
Viral genome sequencing

ABSTRACT

Lumpy skin disease virus (LSDV) is a poxvirus that causes systemic disease in cattle, resulting in substantial economic loss to affected communities. LSDV is a rapidly emerging pathogen of growing global concern that recently spread from Africa and the Middle East into Europe and Asia, impacting the cattle population in these regions. An increase in research efforts into LSDV is required to address key knowledge gaps, however this is hampered by lack of suitable cell lines on which to propagate and study the virus. In this work we describe the replication and spread of LSDV on Madin-Darby bovine kidney (MDBK) cells, and the formation of foci-type poxvirus plaques by LSDV on MDBK cells. Methods utilising MDBK cells to quantify neutralising antibodies to LSDV, and to purify LSDV genomic DNA suitable for short read sequencing are described. These research methods broaden the tools available for LSDV researchers and will facilitate the gathering of evidence to underpin the development of LSD control and prevention programmes.

1. Introduction

Lumpy skin disease virus (LSDV), sheeppox virus (SPPV) and goatpox virus (GTPV) are the three members of the capripoxvirus (CPPV) genus within the poxviridae family. All three capripoxvirus species cause high consequence transboundary diseases in ruminants, resulting in substantial production losses in both endemic and epidemic situations (Bolajoko et al., 2019; Casal et al., 2018; Limon et al., 2020; Molla et al., 2017). LSDV causes disease only in cattle and water buffalo, characterized by multifocal cutaneous nodules up to 2 cm in diameter accompanied by weight loss, fever, reduced milk production and depression. Morbidity varies from 5 to 26 % and mortality from 0.03 to 2 % (Abutarbush et al., 2015; Al-Salihi and Hassan, 2015; Ochwo et al., 2018; Sameea Yousefi et al., 2016; Sevik and Dogan, 2016; Tasioudi et al., 2016).

Lumpy skin disease (LSD) was originally described in southern Africa in the 1920s and subsequently spread throughout the African continent and into the Middle East. During the 2012–2020 Eurasian LSD epidemic, the virus spread into Turkey and then south east Europe, the Caucasus, Russia, Kazakhstan and into India and Bangladesh. This recent and unprecedented expansion of the geographical range of LSDV has highlighted the need for greater research efforts into this rapidly

emerging pathogen, and new and improved methods to underpin these efforts.

LSDV has a narrow range of cell culture permissibility. It has traditionally been grown on primary cells of ruminant origin such as lamb testis cells, fetal bovine muscle and fetal bovine skin cells (Binet et al., 2001; OIE, 2019). However, primary cell lines are prone to contamination, time consuming and expensive to produce, and not compatible with current efforts to reduce the use of animals in science. A continuous ovine testis cell line, OA3.Ts, has been shown to support propagation of LSDV (Babiuk et al., 2007) however this cell line grows slowly, has a limited passage number, and its pestivirus status is unclear. There are also a growing number of reports of LSDV propagation on Madin-Darby bovine kidney (MDBK) cells (Moller et al., 2019; Munyanduki et al., 2020; Wallace and Viljoen, 2002).

This study details the growth and spread of LSDV in MDBK cells, including the formation of foci-type poxviral plaques. We describe new methods to quantify LSDV neutralizing antibodies in bovine sera, and to extract and purify genomic LSDV DNA suitable for short read sequencing.

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<https://doi.org/10.1016/j.jviromet.2020.113943>

Received 18 May 2020; Received in revised form 14 July 2020; Accepted 19 July 2020

Available online 22 July 2020

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2. Materials and methods

2.1. Cells and viruses

MDBK cells (p162) obtained from the European Collection of Authenticated Cell Cultures (Public Health England) were grown in Dulbecco's Modified Eagle's Medium (DMEM; Life Technologies) culture medium supplemented with 100 IU/mL penicillin (Gibco), 100 µg/mL streptomycin (Gibco), and either 10 % or 2.5 % fetal bovine serum (FBS; APS/Life Science Group). Cells were propagated at 37 °C in a 5 % CO₂ incubator.

Primary fibroblast cell cultures were isolated from the ears of 8–12-week-old Holstein Friesian cattle. Three cm² biopsies were taken from the lower ear lobe, washed three times in sterile water and shaved using a disposable sterile scalpel blade (No. 24; Scientific Laboratory Supplies). Using the scalpel blade, the skin was carefully excised from the cartilage, cut into 0.5 cm wide strips and placed in 6-well plates containing culture medium (DMEM supplemented with 100 IU/mL penicillin 100 µg/mL streptomycin and 5 µg/mL amphotericin B (Sigma) and 10 mg/mL Dispase II (Sigma) at a ratio of 3:2 v/v medium:dispase. Tissue samples were then incubated for 17 h overnight at 4 °C. The following day, the epidermis was peeled away from the dermis using sterile forceps, and the dermis placed on a petri dish containing culture medium only and cut into 0.5 cm² square pieces. The tissue pieces were dipped in culture medium supplemented with 10 % FBS and placed into 12-well tissue culture plates with the epidermis side facing down. To each tissue piece, 2–3 drops of culture medium supplemented with 10 % FBS were added and incubated overnight at 37 °C in a 5 % CO₂ incubator. The following day, 8 mL of culture medium supplemented with 10 % FBS was added to each plate. The medium was changed at 48 h and tissue pieces removed from the wells at 72 h. Attached fibroblast cells were cultured to 80–100 % confluence followed by trypsinisation (0.25 % Trypsin-EDTA; Life Technologies) and growth in 6-well plates.

LSDV wildtype was isolated from a diseased animal in Cameroon, Africa, and submitted to the OIE LSDV Reference Laboratory at the Pirbright Institute on the 22/08/2006 (POX-V1-16-06). LSDV Neethling strain is an attenuated vaccine strain and has been described previously (Kara et al., 2003). Unless otherwise specified, the experiments presented here were carried out with LSDV that had been semi-purified through a 36 % sucrose cushion. Briefly, MDBK cells were grown in T175 cm² tissue culture flasks to 80–90 % confluence in culture medium supplemented with 2.5 % FBS. The cells were infected at a low MOI (0.01) and incubated at 37 °C in a 5 % CO₂ incubator for 7 days. The cell pellets were collected, resuspended in 10 mM Tris-HCl buffer (pH 9; Sigma) and lysed using a Dounce homogeniser. Culture supernatants were layered on 18 mL of 36 % sucrose in 32 mL OptiSeal tubes (Beckman Coulter) and centrifuged at 13,500 rpm for 80 min in an Optima™ L-100 XP Ultracentrifuge using a SW-28 rotor (Beckman Coulter). Supernatants were discarded, and virus pellets resuspended in 500 µL of 10 mM Tris-HCl buffer (pH 9) and stored at –80 °C. Purified virus was sonicated for 30 s at ~160 W using a cup horn sonicator (Misonix 3000) before use in downstream assays.

2.2. Antibodies and sera

The polyclonal anti-LSDV074 antibody was a kind gift from IDvet (France). The sera used in the neutralization assays were collected from five calves at 21 days post-challenge with LSDV (Sanz-Bernardo et al., 2020).

2.3. Single-step and multi-step growth curves

Cells were infected with LSDV at a MOI of 5 (single-step) or 0.01 (multi-step) for 1 h at 37 °C in a 5 % CO₂ incubator. The inoculum was removed (time point 0 h) and cells washed three times with culture

medium (DMEM, 100 IU/mL penicillin and 100 µg/mL streptomycin and 2.5 % FBS). At subsequent indicated time points, cells were either scraped into the supernatant or the cell pellet and supernatant were collected separately before being titrated on MDBK cells.

Viral titration was assessed using plaque assay and expressed as number of plaque forming units (PFU)/mL. Briefly, MDBK cells were cultured in 6-well plates to 80–90 % confluence. Tenfold serial dilutions were prepared in culture medium from each time point; cell monolayers were infected with 1 mL of each serial dilution and incubated for 1 h at 37 °C in a 5 % CO₂ incubator. The inocula were then removed, and cells overlaid with a 1:1 solution of 2X DMEM (Merck Millipore) supplemented with 100 IU/mL penicillin and 100 µg/mL streptomycin, 2 % sodium pyruvate, 5 % sodium bicarbonate and 5 % FBS and 3 % carboxymethylcellulose (Sigma). After 7-day incubation at 37 °C in a 5 % CO₂ incubator, the overlay was removed, cells stained with 5 % crystal violet in 20 % methanol (Sigma) for 1 h at RT and washed with tap water. Stained plates were allowed to air dry at RT overnight before plaques were counted microscopically. Virus titer was determined as the number of plaques counted at the highest dilution (PFU/mL).

2.4. Immunofluorescence

MDBK cells were infected with LSDV and incubated at 37 °C in a 5 % CO₂ incubator for 4 days before being fixed with 4 % paraformaldehyde (Sigma) at RT for 15 min. Fixed cells were washed three times with PBS, permeabilized with 0.2 % TritonX100 and washed again before labeling with primary and secondary antibodies. The anti-LSDV074 antibody was diluted 1:500 in blocking buffer (2 % FBS in PBS) and added to cells. After 1 h incubation at 37 °C, cells were washed and incubated again for 1 h with a 1:1000 dilution of a goat anti-rabbit IgG (H + L) antibody (Abcam). Cells were washed three times before coverslips were mounted on slides.

2.5. Immunofluorescent virus neutralisation test (IFVNT)

MDBK cells were seeded in 24-well tissue culture plates (Corning) at a cell suspension of 4×10^5 cells/mL and incubated overnight at 37 °C in a 5 % CO₂ incubator. All test, positive and negative control sera were heat inactivated at 56 °C for 30 min. Each serum was diluted 1:10 in culture medium (DMEM supplemented with 50 IU/mL penicillin, 50 µg/mL streptomycin and 2.5 % heat inactivated FBS). Twofold serial dilutions (1:10 to 1:1280) were then prepared from the test sera. To all serum samples, an equal volume (300 µL) of LSDV Neethling (700 PFU/mL) was added, and samples incubated at 37 °C for 1 h in a 5 % CO₂ incubator. In addition to the serum controls, a mock cell control and a virus only control was prepared. All test, positive and negative control sera were tested in duplicate. 250 µL of the test and control samples were added to MDBK cells at corresponding wells. Cells were incubated at 37 °C in a 5 % CO₂ incubator for 4 days. All cells were fixed with 4 % paraformaldehyde (Sigma) and incubated at RT for 15 min. Cells were washed 3 times with PBS, permeabilised with 0.2 % Triton X-100 (Sigma) and incubated for 5 min at RT before washing again as described. To each well, 250 µL of anti-LSDV074 antibody (IDvet) diluted 1:500 in blocking buffer (2 % FBS in PBS) were added, and cells incubated at 37 °C for 1 h in a 5 % CO₂ incubator. Wells were washed as described and cells incubated at 37 °C for 1 h in a 5 % CO₂ incubator with 250 µL of goat anti-rabbit IgG (H + L) (Abcam) diluted 1:1000 in blocking buffer. Wells were washed as described before counting the number of foci under a fluorescent light microscope (Olympus CKX53). A cut off of 50 foci per/well was used for negative samples. The neutralizing antibody titer was determined as the highest dilution at which no foci were identified indicating complete neutralization.

2.6. Purification and sequencing of LSDV DNA

LSDV was purified using a protocol adapted from (Hughes et al.,

2017). Infected MDBK cells were scraped from T175 cm² flasks and centrifuged at 2000 rpm for 10 min to pellet cells. The supernatants were discarded, and the pellets resuspended in 20 mL of 1 mM Tris-HCl pH9. Suspensions were sonicated 3 times for 1 min in a cup horn sonicator. Samples were vortexed and kept on ice for 30 s between each sonication. To each sample, 5 µL of Benzonase® (> 250 units/µL, Sigma) were added, followed by incubation for 30 min at RT. Samples were centrifuged at 2000 rpm for 10 min, the supernatants were collected and carefully layered over 5 mL of a 36 % sucrose solution in 1 mM Tris-HCl pH9 in a 32 mL OptiSeal tube (Beckman Coulter) and centrifuged at 13,500 rpm for 80 min in an Optima™ L-100 XP Ultracentrifuge using a SW-28 rotor. Supernatants were discarded, pellets resuspended in 3 mL of 1 mM Tris-HCl pH9 and sonicated as above before being layered over 1 mL of a 36 % sucrose in 1 mM Tris-HCl pH9 in a 5 mL OptiSeal tube (Beckman Coulter). Samples were centrifuged at 15,700 rpm for 80 min in a SW-55 rotor (Beckman Coulter), supernatants were discarded, and the pellets resuspended in 500 µL of 1 mM Tris-HCl pH9 in a nuclease-free tube (Eppendorf). Samples were then treated with 33 µL of 1.5 M Tris pH 8.8, 50 µL of 10 % SDS, 100 µL of 60 % sucrose in 1 mM Tris-HCl pH9 and 85 µL of proteinase K (20 mg/mL, Thermo Fisher Scientific) for 4 h at 37 °C, followed by phenol-chloroform extraction and ethanol precipitation. Precipitated DNA was resuspended in a suitable volume of nuclease-free water. DNA concentration was determined using a Qubit™ dsDNA HS Assay Kit (Invitrogen) and read in a Qubit 3.0 fluorometer (Invitrogen). Prior to library preparation, nucleic acid QC was performed using a Bioanalyser 2100 and a high sensitivity DNA kit. Samples were diluted to 0.2 ng/µL in 10 mM Tris-HCl and 5 µL of diluted dsDNA was used for sequencing library preparation using the Nextera XT library preparation kit, automated on a Hamilton NGSstar. After bead normalization, the sequencing library pool was diluted to 12.5 pM and loaded onto an Illumina MiSeq 2 × 150 cycle paired end sequencing run.

2.7. Bioinformatic analysis

Short read sequences from MiSeq were filtered and trimmed, and reads mapped to the host genome, Bos Taurus reference genome ARS-UCD1.2 were identified and removed using BBTool (Bushnell, 2020). Burrows-Wheeler Aligner (BWA) was used to map the remaining reads to the LSDV reference genome (KX894508.1) using (BWA), from which coverage was estimated (BBTools). BCFtools (Danecek and McCarthy, 2017), Freebayes (Garrison and Marth, 2012) and Mosdepth (Pedersen and Quinlan, 2018) were used to generate the consensus genome sequence, and each genome was annotated with the reference genome annotation using Genome Annotation Transfer Utility (GATU) (Tcherepanov et al., 2006).

3. Results

3.1. LSDV forms foci on MDBK cells

In order to determine the permissivity of MDBK cells for LSDV, a wildtype strain isolated from clinical material was inoculated onto MDBK cells, and the cells examined microscopically. Typical poxvirus cytopathic effect (CPE) was noted (Sanderson et al., 1998), characterized by cells becoming rounded and refractile then developing irregular shapes with long, thin projections (Fig. 1A and B). No secondary plaques forming comet tails, as seen in vaccinia virus infected cell cultures under liquid overlay (Law et al., 2002), were seen.

At 7 d post inoculation the cells and supernatant were collected together and LSDV semi-purified by breaking open the cells, clarifying the lysate, and centrifuging it through a 36 % sucrose cushion. The semi-purified virus stock was then titrated in a ten-fold dilution series on to MDBK cells. After incubation for 7 d cells were examined microscopically. Multifocal areas of hyperplastic cells, approximately 250–300 cells in each focus, were scattered throughout the wells. The

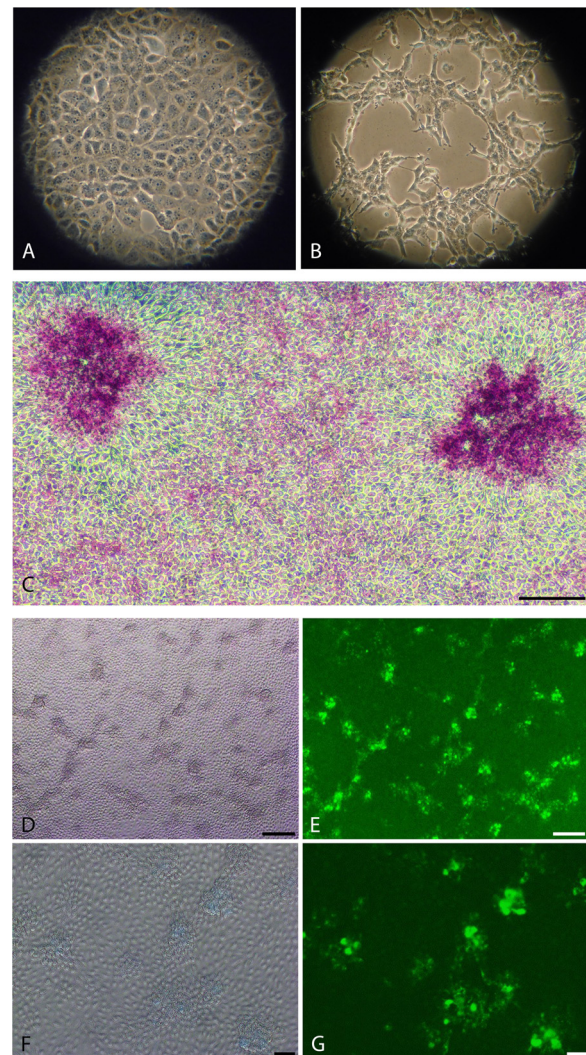


Fig. 1. LSDV forms foci on MDBK cells. (A–B) MDBK cells were mock infected (A) or infected with LSDV (B) at a MOI of 5 then incubated for 24 h. (C) LSDV was titred on to MDBK cells and incubated for 7 days before being stained with crystal violet stain to highlight viral foci. Scale bar = 200 µm. (D–G) LSDV was titred on to MDBK cells and incubated for 7 days before being labelled with antisera raised against LSDV074. Corresponding brightfield (D and F) and fluorescent (E and G) images are shown. The scale bar in D and E is 200 µm, in F and G 50 µm.

foci were visible under bright field and enhanced by crystal violet staining (Fig. 1C). The number of foci in each well decreased by a factor of ten in accordance with the dilution series. No foci were present in uninfected wells.

In order to confirm that the hyperplastic foci contained LSDV-infected cells a rabbit polyclonal antibody raised against LSDV074 (the predicted LSDV orthologue of the vaccinia virus protein H3) (Tulman et al., 2001) were used to label LSDV infected cells. Briefly, MDBK cells were infected with LSDV at a low MOI in order to produce individual foci. Cells were then permeabilised, antibody added, and labelled cells visualised using fluorescence microscopy. As seen in Fig. 1D–G the hyperplastic foci were labelled with the anti-LSDV074 antibody. No labelling was identified in MDBK cells that were not part of a foci. This indicates that the foci that form on MDBK cells in response to LSDV infection are composed of LSDV-infected cells.

3.2. LSDV replicates to high titers on MDBK cells

In order to compare virus replication a wildtype strain of LSDV was

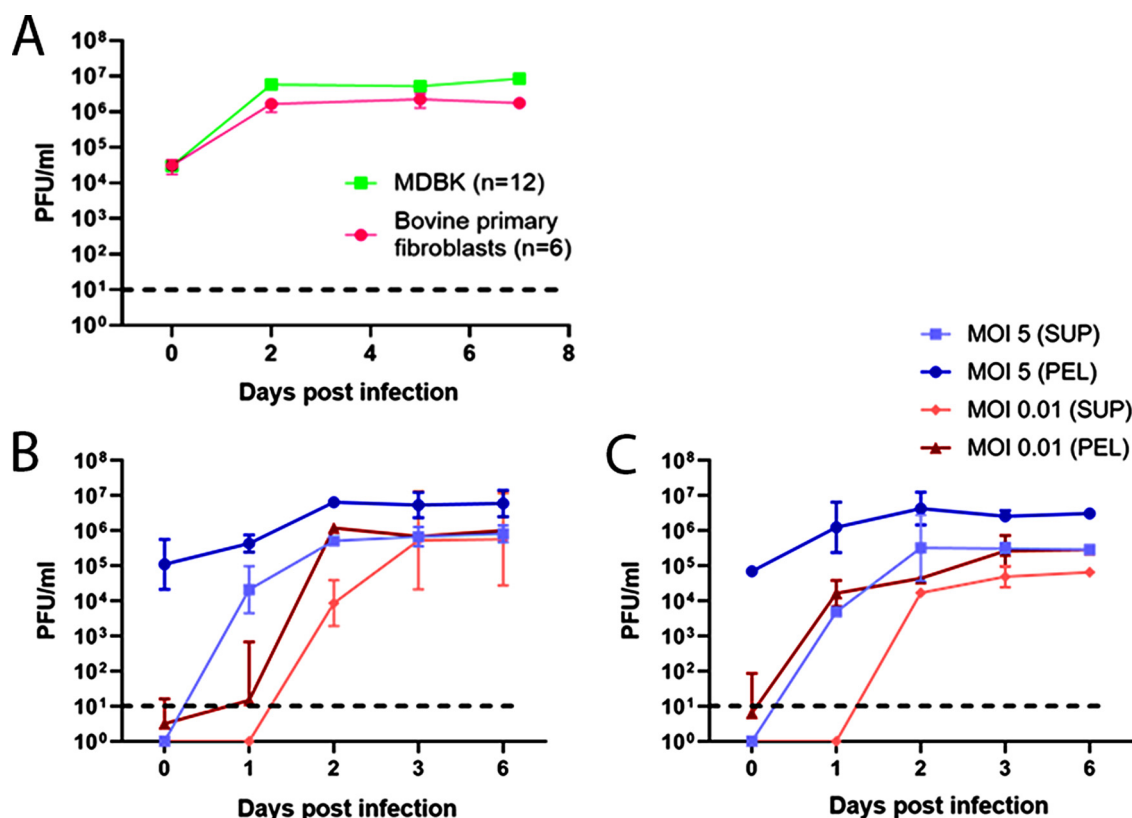


Fig. 2. LSDV replicates and spreads on MDBK cells. (A) MDBK cells ($n = 12$) or bovine primary fibroblasts ($n = 6$) were inoculated with LSDV (MOI = 5). At the timepoints shown cells were scraped into the supernatant and virus titred on MDBK cells. Values shown are means, error bars are SEM. (B and C) MDBK cells were inoculated with LSDV wildtype (B) or LSDV Neethling (C) at a MOI of 5 (one-step growth curve) or MOI = 0.01 (multi-step growth curve). At the timepoints shown virus loads in the cell associated (PEL) and supernatant (SUP) fractions were determined by plaque assay on MDBK cells. Values are the mean of two biological replicates, error bars = SEM.

inoculated on to MDBK cells and primary bovine fibroblasts at a MOI of 5 and incubated for 7 d (Fig. 2A). Cells and supernatant were collected together at the indicated times post infection and titred on MDBK cells. As reported previously, LSDV growth was detected on bovine primary fibroblasts with approximately $1.5\log_{10}$ growth rate detected by day 2 post infection. LSDV also replicated well on MDBK cells with approximately $2.5\log_{10}$ growth rate by day 2 post infection. This indicates that MDBK cells are able to support growth of LSDV to a higher titer than primary bovine fibroblasts.

The growth of LSDV on MDBK cells was then examined in more detail. Wildtype and attenuated (Neethling) strains of LSDV were inoculated on to MDBK cells at a low (0.01) and high (5) MOI. A low MOI results in a multi-step growth curve that measures multiple rounds of virus replication and virus spread, while the high MOI infection is a single-step growth curve that measures just one cycle of virus replication. For both growth curves supernatant and cell pellet were collected separately at the indicated time points and titred on MDBK cells (Fig. 2B and C). The cell pellet was predicted to contain predominantly intracellular mature virions (IMVs) while the supernatant would contain both extracellular enveloped virions (EEVs) and IMVs that had been released from lysed cells. In both growth curves in both cellular and supernatant fractions, the two virus strains tested showed similar growth kinetics. The amount of virus present in the cellular fraction of the LSDV single-step (MOI = 5) growth curve increased by $2\log_{10}$ PFU/mL over 2 d. LSDV was first detected in the supernatant of the one-step growth curve at 1 d post infection and then increased by over $5\log_{10}$ PFU/mL at 3 d post infection. The amount of LSDV in the multi-step growth curve increased by over $5\log_{10}$ over 2–3 d in both the cellular and supernatant fractions. The amount of virus detected in the cellular fraction of the attenuated virus strain was higher than the wildtype

strain at 1 d post infection, however this difference was not apparent at later timepoints.

3.3. Development of an immunofluorescent virus neutralization test (IFVNT)

The identification of LSDV foci formation on MDBK cells (Fig. 1C) suggested a plaque reduction neutralization test (PRNT) could be developed. A PRNT is a standard method used for detection of orthopoxvirus neutralizing antibodies (Putz et al., 2006). In order to develop a PRNT for LSDV, sera from five calves experimentally inoculated with LSDV were analysed. The experimental study has been described previously (Sanz-Bernardo et al., 2020). Briefly, one calf (#1) was not inoculated and served as a negative control. The remaining four animals were inoculated with 3×10^6 PFU of wildtype LSDV. Two calves developed multifocal cutaneous nodules characteristic of LSD (#3 and #5) while two calves (#2 and #4) remained clinically normal. In order to quantify the neutralizing antibodies present in the sera at 21 days post inoculation LSDV Neethling (2.1×10^2 PFU) was mixed with serial dilutions of the sera. The dilutions were then mixed with MDBK cells and plated on a 24-well plate. After 4 days the wells were labelled with anti-LSDV074 antibody and an appropriate fluorescently-labelled secondary antibody and the foci counted (Fig. 3). Greater than 50 LSDV foci were present even at the lowest serum dilution of 1:10 in the non-inoculated control animal (calf #1), indicating that no neutralizing antibodies were present in the sera. In contrast neutralizing antibodies were detected in sera from all four inoculated animals (calves #2–#5). Only a small number of foci formed after virus was added to sera from the two clinical calves (#3 and #5) even at the highest sera dilution (1:1280). In comparison greater than 50 foci were present at this

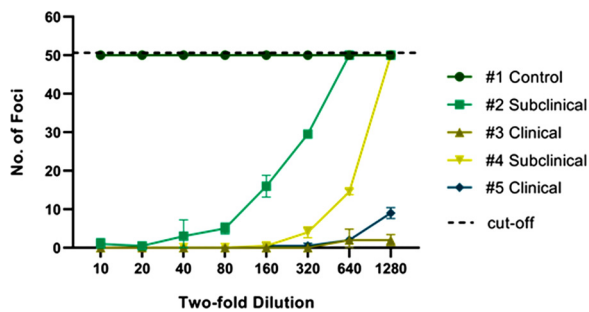


Fig. 3. Quantification of neutralizing antibodies to LSDV using an immunofluorescent virus neutralization test (IFVNT). Serum collected at 21 days post inoculation from five calves experimentally challenged with LSDV was tested by IFVNT. Individual fluorescent foci were counted (using 50 foci as a maximum value) at each dilution and plotted. The clinical outcome of the LSDV inoculation in each calf is noted on the right of the figure. Calf #1 was a non-inoculated in-contact control.

dilution when sera from the two subclinical calves (#2 and #4) were examined. The neutralization titre, measured as the highest dilution at which total neutralization occurs, was 320 for calves #3 and #5, and 160 for calf #4 and 20 for calf #2. Overall, the IFVNT was quicker to carry out and less subjective than the traditional serum neutralization test. It discriminated between inoculated and non-inoculated calves and revealed that the two clinical calves developed higher titers of neutralizing antibodies than the two subclinical calves.

3.4. Optimization of a method for purifying LSDV genomic DNA for whole genome sequencing

Sequencing of the entire genome of poxviruses is used for molecular epidemiological studies and to compare the evolution of viruses over time. We used MDBK cells to adapt a published protocol for variola virus purification suitable for use in high-containment laboratories (Hughes et al., 2017). Briefly MDBK cells infected with LSDV were lysed by sonication in a hypotonic buffer, treated with Benzonase® and virus particles purified through sequential sucrose cushion ultracentrifugations, followed by protease digestion of virus cores and phenol-chloroform extraction and ethanol precipitation of viral DNA. The concentration of DNA ($n = 14$) recovered from $1 \times \text{T175cm}^2$ flask of original starting material was $6.99 \text{ ng}/\mu\text{L}$ in a total volume of $100 \mu\text{L}$ as measured by the Qubit. Sequencing libraries were generated using 1 ng of dsDNA in a $5 \mu\text{L}$ input volume using the Nextera XT library preparation kit. Libraries were checked by Bioanalyser 2100 using a high sensitivity DNA kit at the post amplification stage before continuing to normalization. The final normalized pool of libraries was sequenced using an Illumina MiSeq on a 2×150 cycle paired end run. Analysis of data from three experiments showed that after the removal of adaptors and lower quality reads, 34–41 % of the sequencing reads mapped to the host genome. After the removal of reads that mapped to the host genome, an estimated 32–148 fold coverage was achieved when mapped against LSDV genome, covering 98.9–99.95 % of the genome in the three runs. GATU was able to annotate all genes from the reference genome annotation on each of the consensus sequence genomes generated from the alignment, with an average of 97.7–99.9 % protein similarity.

4. Discussion and conclusions

There are few cell lines described in the literature for propagating capripoxviruses including LSDV. LSDV has traditionally been grown on primary cell lines such as lamb testis cells or primary bovine fibroblasts or the ovine testes cell line OA3.Ts. However primary cells and OA3.Ts are slow to grow, have a restricted growth passage number, and are prone to contamination. We examined MDBK cells as an alternative cell

line for LSDV propagation.

MDBKs are a ruminant adherent cell line originating from the bovine kidney and showing epithelial characteristics. This cell line was chosen for its bovine origin, rapid growth characteristics, and amenability to CRISPR/Cas9 modification. Our research showed LSDV produces characteristic poxvirus CPE in infected MDBK cells including the formation of distinctive elongated cellular projections. When titrated on MDBK cells, LSDV formed foci-type plaques similar to that formed by the leporipoxviruses Shope fibroma virus (Sabourdy et al., 2004) and myxoma virus (Irwin and Evans, 2012), and the orthopoxviruses taterapoxvirus and variola virus minor (Lourie et al., 1975). In contrast, poxviruses such as vaccinia virus and cowpox virus typically produce a viral plaque that has a centrally cleared area surrounded by a rim of cells exhibiting CPE. The mechanisms underpinning the type of plaque formed by poxviruses are not fully understood, however inserting the VACV F11 L ORF into the myxoma virus genome resulted in a change from a foci to a cleared plaque phenotype, suggesting the F11 protein influences the plaque morphology of poxviruses (Irwin and Evans, 2012).

A plaque-based titration assay for LSDV was developed and used to compare growth of LSDV on MDBK cells and primary bovine fibroblasts. LSDV was found to grow to slightly higher titers in MDBK cells, indicating that they are suitable for propagating LSDV. Detailed characterization of the growth kinetics of wildtype and vaccine strains of LSDV on MDBK cells found approximately $2\log_{10}$ PFU/mL increase in virus over two days in a one-step growth curve and $5\log_{10}$ PFU/mL increase over three days in a multi-step growth curve. Virus increase was seen in both the cell pellet and the supernatant suggesting that LSDV virions are released as EEV, however the virions detected in the supernatant could also represent IMVs released from lysed cells. Antibodies that specifically neutralize IMVs of LSDV are required in order to investigate this further.

The attenuated (Neethling) strain of LSDV is widely used as a live-attenuated vaccine for the control of LSD. The growth of this strain to high titres on MDBK cells suggests that MDBK cells are suitable for use in the production of live-attenuated LSDV vaccines. The growth of a wildtype strain of LSDV also to a high titre in MDBK cells suggests MDBK cells are also suitable in a diagnostic setting for isolation of LSDV from clinical samples.

The recommended method for detecting neutralizing antibodies to LSDV is currently the serum neutralization test (SNT) using a read-out of tissue culture infectious dose (TCID_{50}) (OIE, 2019). This method is often used for viruses which do not form easily identifiable plaques in tissue culture. The sera dilution at which 50 % of replicates (usually wells or plates) exhibit cytopathic effect is designated the TCID_{50} (Reed and Muench, 1938). We developed a plaque-based Immunofluorescent Virus Neutralization Test (IFVNT) by adapting previously published methods for quantification of neutralizing antibodies to other poxviruses. The IFVNT incorporated a fluorescence-based detection method instead of the traditional crystal violet stain. Foci were labelled using an anti-LSDV074 antibody coupled with a fluorescently tagged anti-bovine secondary antibody and visualised with epifluorescence microscopy. The fluorescently-labelled foci were easier and quicker to count compared to crystal violet-stained foci, and foci quantification was less subjective than detection of CPE in a traditional SNT. The IFVNT was shown to detect neutralizing antibodies in four experimentally-inoculated calves, including two animals which did not develop clinical disease. The ability to count individual foci at different sera dilutions enables endpoint neutralization titers to be determined, but also allows for more discriminating measurements to be made such as the PRNT_{50} value which is the concentration of serum to reduce the number of plaques by 50 % compared to the serum free virus. An assay using a peroxidase staining methodology has recently been developed for the detection of both neutralizing and total binding antibodies to LSDV by Immunoperoxidase Monolayer Assay (IPMA) on OA3.T cell lines (Hageman et al., 2019). Detection of neutralizing antibodies provides

information about the function of antibodies present in the sera and can be used to extrapolate levels of protection in the animal, which is the major advantage of the assay over ELISA-based methods for detecting a serological response to LSDV. However, the neutralization assays remain unsuited to a high throughput format as they are more time consuming and technically challenging compared to a simple ELISA. Neutralization assays remain more suited to research purposes rather than, for example, post-vaccination monitoring.

MDBK cells were also used to develop a protocol for preparation of DNA for whole genome sequencing of LSDV. The starting material was treated with benzonase and the LSDV genomic DNA was extracted from cell lysates partially purified through sucrose cushion ultracentrifugation. Viral genome DNA extraction from a crude extract of infected cells usually results in low quality DNA, high host DNA contamination and consequently reduction in the length and number of reads obtained from a single viral genome sequencing run. Our protocol is inexpensive, rapid and allowed recovery of sufficient genomic viral DNA for short read sequencing using Illumina platforms from just one LSDV-infected 175 cm² flask of MDBK cells. Whilst some degree of host-genome contamination was observed, an average of 32–148 fold coverage was achieved, allowing variant calling in 98.9–99.95 % of the genome (short-read sequencing was unable to resolve the highly repetitive regions at the ends of LSDV).

The rapid geographic expansion of LSD into Europe and Asia since 2012 has increased its impact on global agriculture and resulted in more research laboratories studying LSDV. This paper provides details of new methods which will improve the breadth of research into LSDV and promote standardization of protocols across laboratories. The other two species of the capripoxvirus genus are SPPV and GTPV, both important pathogens of small ruminants in low and middle income countries in Africa and Asia. Initial studies have indicated that these viruses also grow to high titers on MDBK cells and form foci similar to LSDV. The protocols described in this paper are therefore likely to be easily adapted for GTPV and SPPV.

Funding

This work was supported by the Biotechnology and Biological Sciences Research Council (BBSRC) responsive mode research grants BB/R002606/1 and BB/R008833/1, and strategic funding from the BBSRC to the Pirbright Institute (BBS/E/1/00007037, BBS/E/1/00007033, BBS/E/1/00007036, BBS/E/1/00007039, BBS/E/1/00002087) and the Roslin Institute (BBS/E/D/20002173). This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 773701.

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Petra C. Fay: Conceptualization, Investigation, Methodology, Data curation, Formal analysis, Writing - original draft, Writing - review & editing. **Charlotte G. Cook:** Investigation, Methodology, Formal analysis, Writing - original draft, Writing - review & editing. **Najith Wijesiriwardana:** Investigation, Methodology, Formal analysis, Writing - original draft, Writing - review & editing. **Gessica Tore:** Conceptualization, Investigation, Formal analysis, Writing - original draft, Writing - review & editing. **Loic Comtet:** Conceptualization, Funding acquisition, Supervision, Writing - review & editing. **Alix Carpentier:** Methodology, Resources, Writing - review & editing. **Barbara Shih:** Data curation, Investigation, Formal analysis, Writing - review & editing. **Graham Freimanis:** Resources, Writing - review & editing. **Isamar R. Haga:** Formal analysis, Methodology, Investigation, Supervision, Writing - original draft, Writing - review & editing. **Philippa M. Beard:** Conceptualization, Funding acquisition, Supervision, Project administration, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors have no competing interests to declare.

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